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14. ABSTRACT

The PI3K/AKT pathway is one of the most deregulated pathways in breast cancers (>70%), and a major contributor to tumor progression. PI3Ks and AKTs comprise of multiple isoforms that play a critical role in a wide variety of physiological progresses. Moreover, during cancer progression, different PI3K and AKT isoforms may have different and even opposite roles. Notably, PI3Kβ and AKT2 have been identified as the major isoform that contribute to breast cancer growth and metastasis. Yet, it is not yet clear how to specifically target the PI3Kβ/AKT2 without causing wide spread side effects. In this proposal, we aim to test the hypothesis that WDR26 functions as a novel regulator of the PI3K□/AKT2 pathway, and a previously unidentified marker/therapeutic target in advanced breast cancer, in particular, triple negative breast cancer (TNBC). Our results thus far demonstrated that WDR26 serves as a scaffold that fosters the interaction between Gβγ, PI3Kβ, and AKT2; and in highly malignant and invasive breast tumors, upregulated WDR26 overactivates the PI3Kβ/AKT2 pathway, promoting breast tumor growth and metastasis.
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1. **Introduction:**

Breast cancer is the second most common cause of cancer death in women in the US. Despite recent advances in the development of new treatments (e.g., targeted therapies) limited options are available for patients with advanced breast cancer, in particular, triple negative breast cancer (TNBC). Thus, it is imperative to develop novel approaches for treating advanced breast cancer.

In over 70% of breast cancers, the PI3K/AKT signaling pathway is dysregulated. This pathway transmits signals downstream from critical cell surface receptors, including receptor tyrosine kinase and G protein-coupled receptors (GPCRs) and, when dysregulated, is believed to promote tumor progression, resistance to available therapies and cancer relapse. PI3K/AKT signaling plays a central role in driving many aggressive breast cancers, making it one of the most hotly pursued therapeutic targets for new breast cancer treatments. However, ongoing concerns remain regarding the efficacy and long-term safety of directly inhibiting enzymatic activities that control a wide spectrum of biological processes. Thus, it is imperative that new strategies be developed for regulating, with a high degree of specificity, the signals this pathway emits, so we can harness the power of the PI3Ks and AKTs to control breast cancer.

Our preliminary studies suggest that WDR26 may function as a scaffold that fosters the interaction between Gβγ, PI3Kβ, and AKT2; and in highly malignant and invasive breast tumors, upregulated WDR26 overactivates the PI3Kβ/AKT2 pathway, promoting breast tumor growth and metastasis. Moreover, WDR26 may serve as a previously unidentified, yet powerful prognostic marker /therapeutic target for advanced TNBC patients. In this proposal, we aim to define precisely the key role of WDR26 in breast tumor (in particular TNBC) development, as well as to determine, using preclinical models of advanced triple negative breast cancer, the therapeutic efficacy of targeting WDR26 with small molecule inhibitors. Our proposed studies could potentially uncover a novel and efficacious approach for developing a new PI3K/AKT-targeted therapy that would improve the outcome for patients affected by advanced breast cancer (in particular, triple negative breast cancer), including the women in the military services. This could be a major breakthrough both in our understanding of the molecular mechanisms that drive TNBC progression and in our effort to eliminate suffering and death caused by advanced breast cancer.

2. **Keywords:** Breast cancer growth and Metastasis, heterotrimeric G protein βγ subunits, G protein-coupled receptors, signal transduction, PI3K, AKT

3. **Accomplishments:** Summarized below are the accomplishments from research work performed in the 1st yr of this project in direct alignment with the Statement of Work (SOW) schedule.

Task/Milestone 1. Determine how WDR26 promotes PI3K/AKT activation and breast tumor progression. This aim will determine how WDR26 promotes tumor growth and metastasis via dysregulation of the PI3Kβ/AKT2 pathway. (months 1-24)

Major Goal 1: Assess how WDR26 regulates PI3K/AKT signaling in breast cancer cells. (months 1-12)

1a. Determine WDR26 levels in a panel of breast cancer cell lines. (months 1-4).

Accomplishments: we have determined WDR26 expression in MDA-MB231, DU4475, BT549, MCF7, T47D and MCF10A cell lines. We found that WDR26 is upregulated in triple-negative breast cancer cell lines MDA-MB231, DU4475 and BT549, but not in estrogen receptor positive cell lines MCF7 and T47D, as compared to the non-transformed epithelial cell line MCF10A (Fig. 1). We have not yet confirmed these findings in a larger set of breast cancer cell lines as there was a delay in obtaining these cell lines from ATCC in our cell core facility. We anticipate to complete these studies during the second report period.

1b. Evaluate the role of WDR26 in regulating PI3Kβ/AKT2 activation. (months 3-9)

Accomplishment: we have shown that downregulation of WDR26 in MDA-MB231, DU4475 or BT549 impaired cell growth (Fig. 2A-D), migration (Fig. 3A-B) and invasion (Fig. 3C). Conversely,

overexpression of WDR26 in MCF7 cells resulted in enhanced cell growth and migration (Fig. 2E and Fig. 3D). Moreover, we showed that WDR26 is required for PI3K/AKT activation as downregulation of WDR26 selectively alleviated GPCR-stimulated AKT phosphorylation (Fig. 4A-B), while overexpression of WDR26 had opposite effects (Fig. 4C).

1c. Dissect how the WDR26 scaffold facilitates Gβγ-mediated PI3Kβ2/AKT2 activation (months 6-12). Accomplishment: we showed that WDR26 selectively co-immunoprecipitated with endogenous Gβγ, PI3Kβ and AKT2 in MDA-MB231 cells (Fig. 5A-B). WDR26 binds AKT2 and enhances its interaction with Gβγ (Fig. 5C). WDR26 also binds PI3Kβ and forms a trimeric complex with PI3Kβ and Gβ1γ2 (Fig. 5D). We also identified the binding sites of PI3Kβ (Fig. 5E) and AKT2 (Fig. 5F) on WDR26. Finally, we showed that disruption of the complex formation between WDR26, Gβγ, PI3Kβ and AKT2 in MDA-MB231 cells impaired AKT activation, tumor cell growth and migration (Fig. 6).

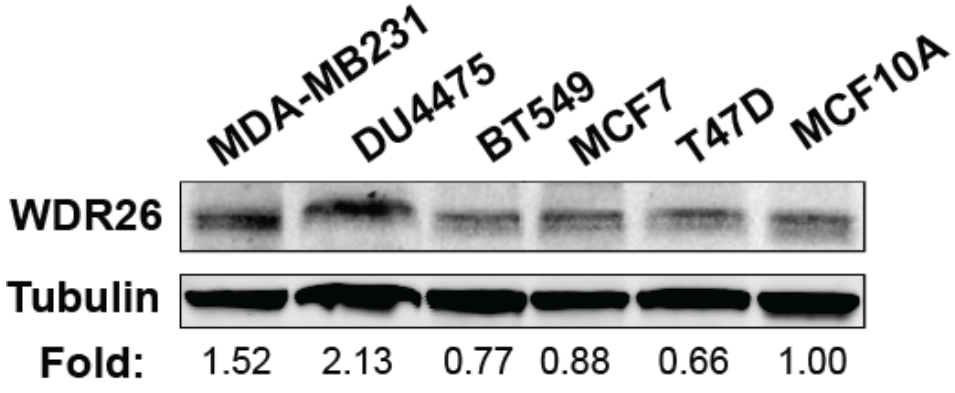

Figure 1. WDR26 is overexpressed in highly malignant breast cancer cell lines Western blot analysis of WDR26 expression in human breast cancer cell lines. The relative level of WDR26 expression is expressed as a fold change over that in MCF10A (after normalization by tubulin) and indicated underneath the blot.

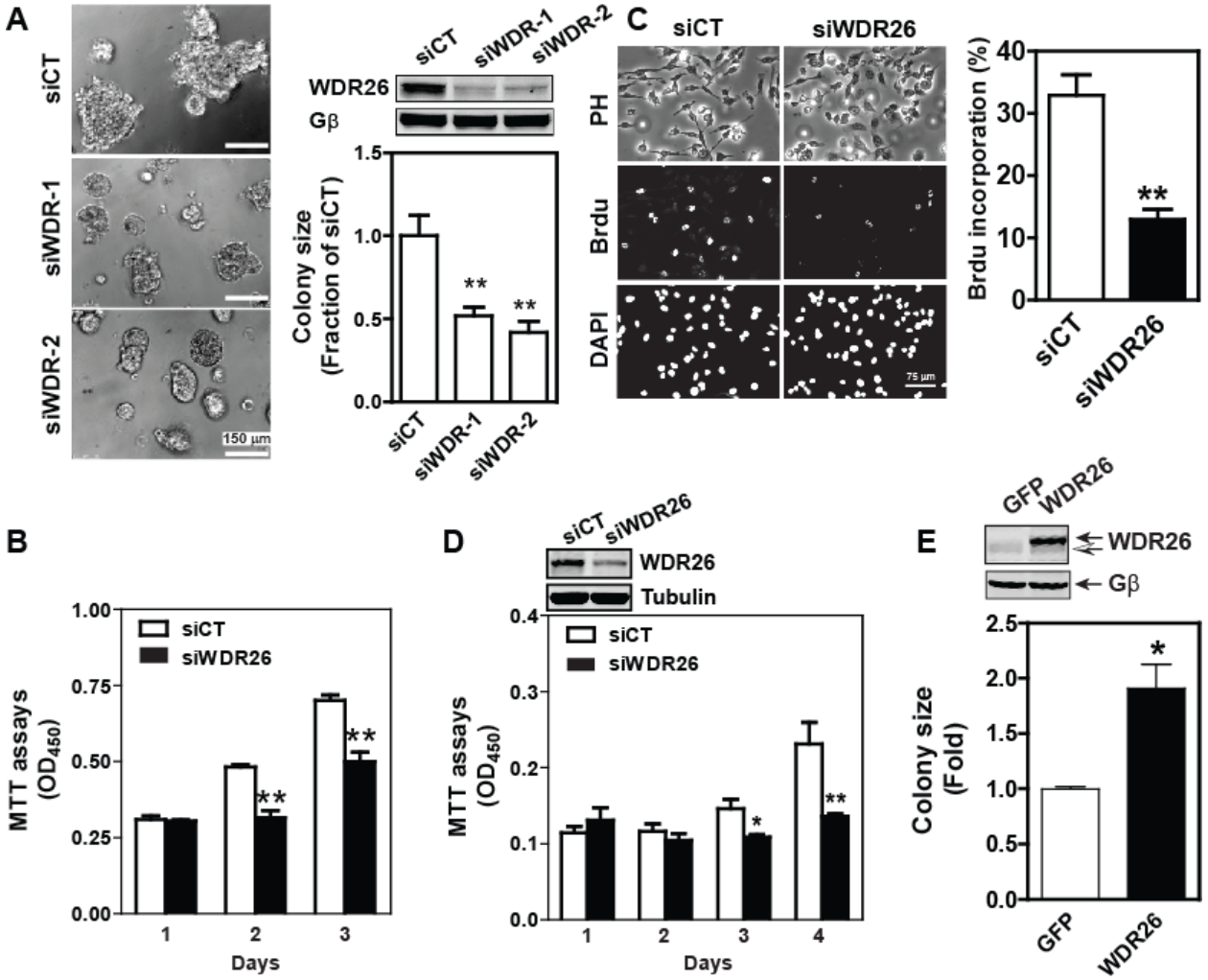

Figure 2. WDR26 promotes proliferation of breast cancer cells. A-D, Downregulation of WDR26 inhibited breast cancer cell growth. MDA-MB231 (A-C) or DU4475 (D) cells were transiently transfected with a control (siCT) siRNA or siRNAs targeting two distinct regions of WDR26 (siWDR-1 and siWDR-2) (A) or siWDR-1 (siWDR26) (B-D). The effect on cell growth in Matrigel (A) was determined by phase-contrast imaging, followed by quantification of the size of the colonies. Colony size is expressed as the fraction of that derived from siCT-transfected cells. The effect on cell growth in 2D culture was measured by MTT assays (B and D) or BrdU incorporation assays (C). BrdU incorporation was detected by immunofluorescence staining. Phase contrast (PH) images of cells and fluorescence images of BrdU and nuclear (DAPI) staining are shown in C. Quantitative data showing the level of BrdU incorporation are expressed as the percentage of cells stained with BrdU. *, ** $p<0.05$ and 0.01 *versus* siCT, respectively (N=4-5). *Insert*, representative blots showing WDR26 downregulation. E, overexpression of WDR26 promotes MCF7 cell growth. MCF7 cells stably expressing inducible GFP or WDR26 were treated with doxycycline (1 μg/ml) and cultured in Matrigel for ten days. The size of cell colonies was quantified and expressed as a fold-increase over that of GFP-expressing colonies. * $p<0.05$ *versus* GFP (n=4). *Insert*, a representative blot showing the level of WDR26 overexpression. Solid and hollow arrows indicate the ectopic and endogenous WDR26 expression in MCF7 cells, respectively.

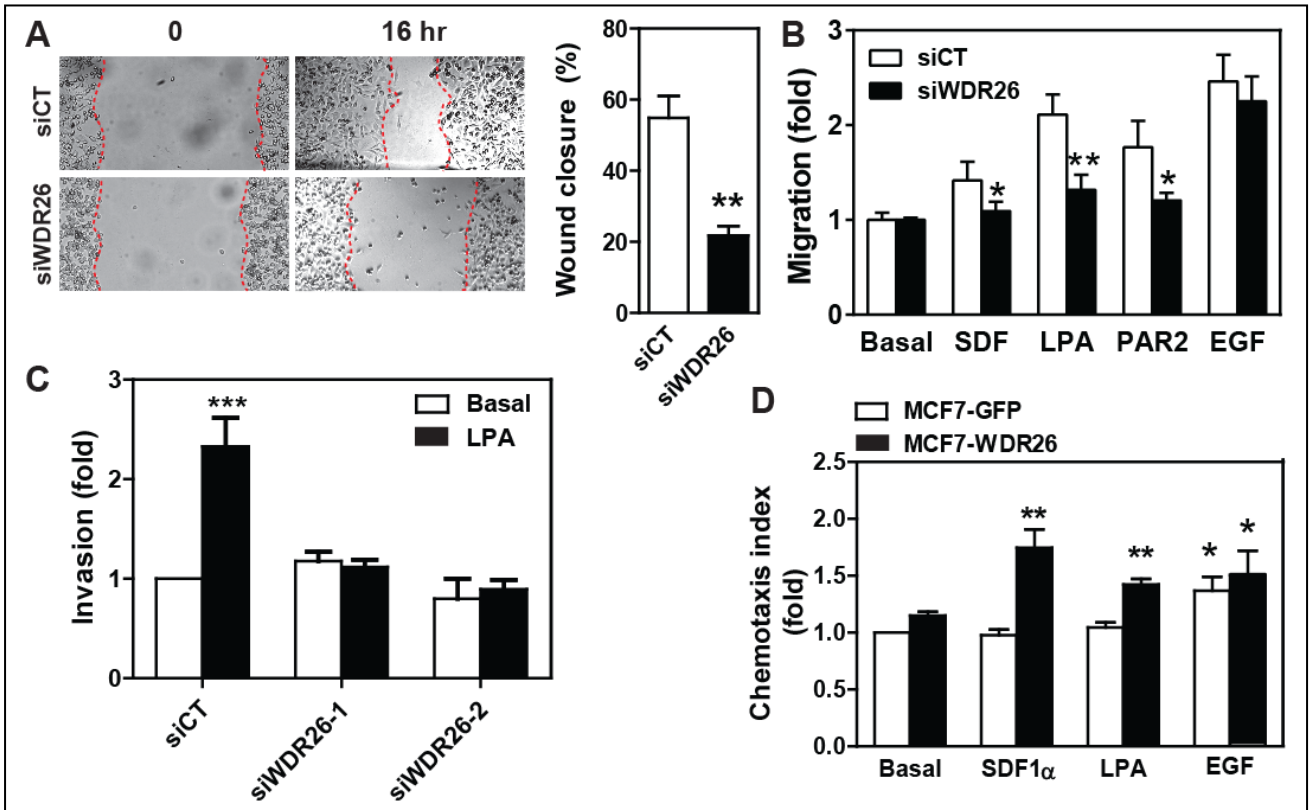

Figure 3. WDR26 promotes breast cancer cell migration and invasion. A-C, MDA-MB231 cells were transiently transfected with a control siRNA (siCT) or siRNAs against WDR26 (siWDR26, siWDR26-1 or siWDR26-2). The effects on cell migration were determined by a wound healing assay (A) and transwell migration assay (B) in response to buffer (basal) or chemoattractants, SDF1α (100 nM), LPA (50 nM), PAR2 agonist peptide (10 μM) and EGF (50 ng/ml). The effect of LPA on cell invasion was determined in a transwell migration assay with the filter coated with a thin layer of Matrigel (C). *, **, *** $p<0.05$, 0.01 and 0.001, respectively, *versus* siCT (N=4-6). D, the effect of WDR26 overexpression on MCF7 cell migration. MCF7 cells stably expressing inducible GFP or WDR26 were treated with doxycycline (1 μg/ml) for 5 days and then subjected to the transwell migration assay as described in B. *, ** $p<0.05$ and 0.01, respectively, *versus* basal (n=4).

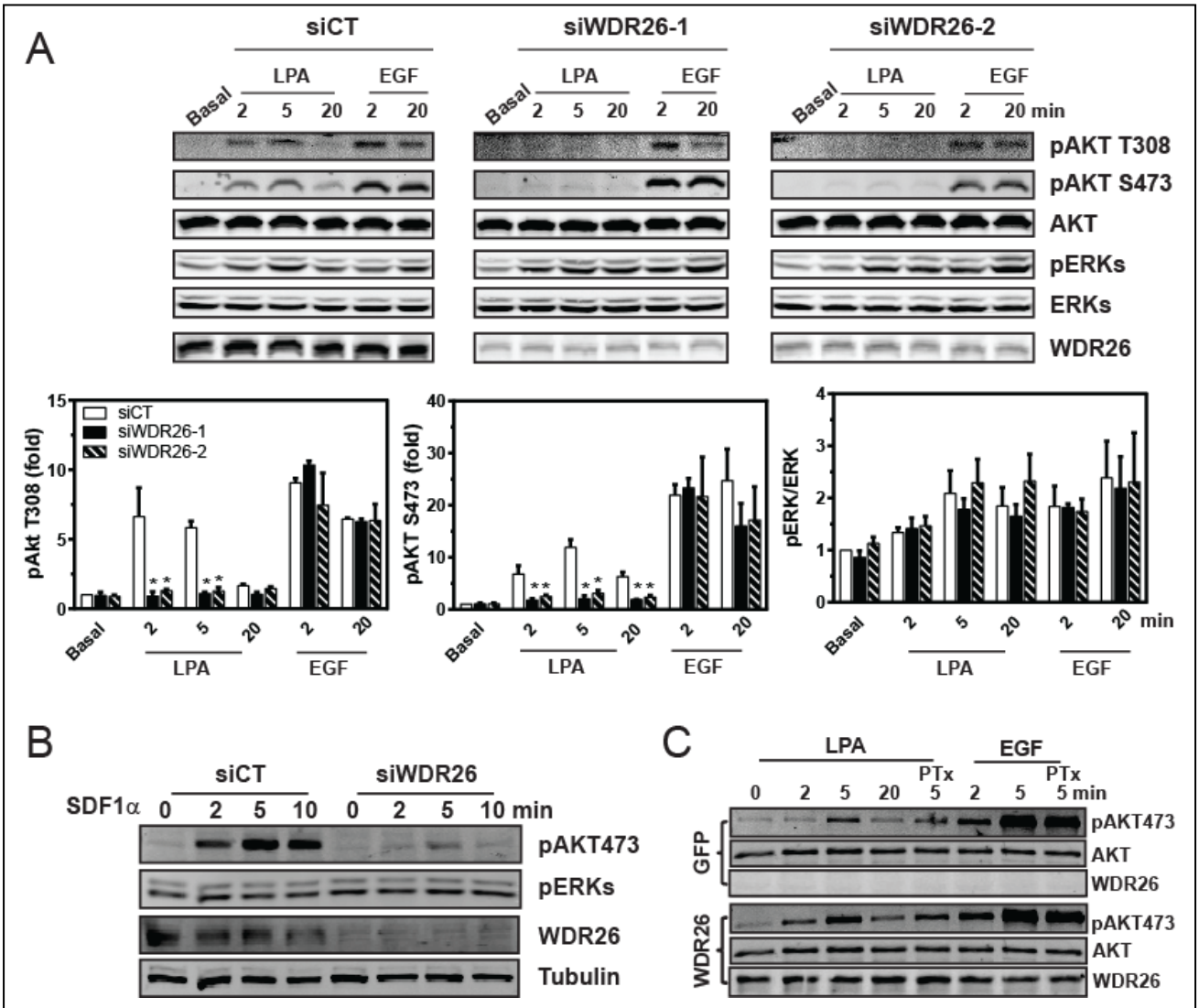

Figure 4. WDR26 selectively regulates Gβγ-mediated AKT phosphorylation. A-B, MDA-MB231 cells were transiently transfected with a control siRNA (siCT) or siRNAs targeting WDR26 (siWDR26), and the effects on LPA (10 μM)-, EGF (50 ng/ml) (A)- or SDF1α (100 nM)(B)-stimulated AKT and ERK phosphorylation were determined by Western blotting. Data in A are expressed as the fold-increase over the unstimulated control (basal) after the levels of phosphorylated proteins are normalized to that of total proteins. *, ** $p<0.05$, *versus* siCT (n=5). C, Western blot analysis of AKT phosphorylation in MCF7 cells expressing inducible GFP or WDR26. To determine the effect of PTx, cells were pre-treated with PTx (200 ng/ml) overnight.

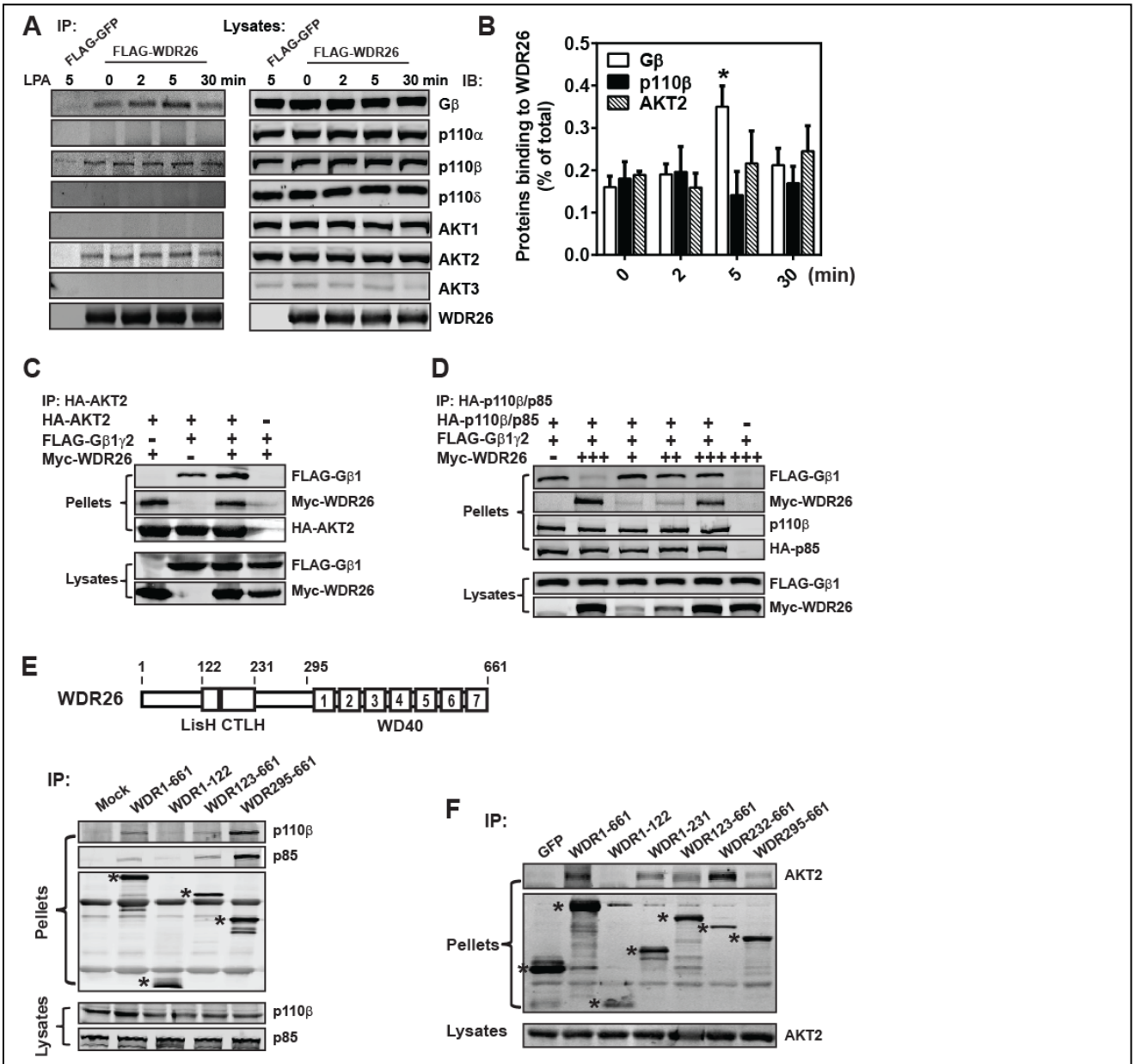

Figure 5. WDR26 fosters the interactions between Gβγ, PI3Kβ and AKT2. A-B, WDR26 is selectively co-immunoprecipitated with endogenous Gβγ, PI3Kβ and AKT2 in MDA-MB231 cells. MDA-MB231 cells expressing FLAG-GFP or FLAG-WDR26 were stimulated with LPA (10 μM) for the indicated time and subjected to immunoprecipitation with an anti-FLAG antibody. Co-immunoprecipitated proteins were detected with the indicated antibodies. Representative blots are shown in A and quantitative data from three independent experiments are shown in B. The amount of immunoprecipitated proteins is expressed as the percentage of total proteins in the lysates, with the background binding from FLAG-GFP immunoprecipitates substracted. * $p<0.05$ *versus* 0 min. C, WDR26 binds AKT2 and enhances its interaction with Gβγ. HEK293 cells were transiently transfected with HA-AKT2 and FLAG-Gb1g2 or Myc-WDR26, or FLAG-Gb1g2 plus Myc-WDR26. Cell lysates were immunoprecipitated with an anti-HA antibody. D, WDR26 binds PI3Kβ and forms a trimeric complex with PI3Kβ and Gβ1γ2. MDA-MB231 cells transduced with adenoviruses encoding the indicated proteins were subjected to co-immunoprecipitation assays as described in C. E-F, the binding sites of PI3Kβ and AKT2 on WDR26. FLAG-GFP, FLAG-WDR26 and its deletion mutants were transiently co-expressed with PI3Kβ in MDA-MB231 (E) or AKT2 in HEK293 (F) cells, and were immunoprecipitated from cell lysates with an anti-FLAG antibody. The bands corresponding to GFP, WDR26, and its mutants are indicated by asterisks. A schematic representation of the WDR26 structure is shown in the top panel of E.

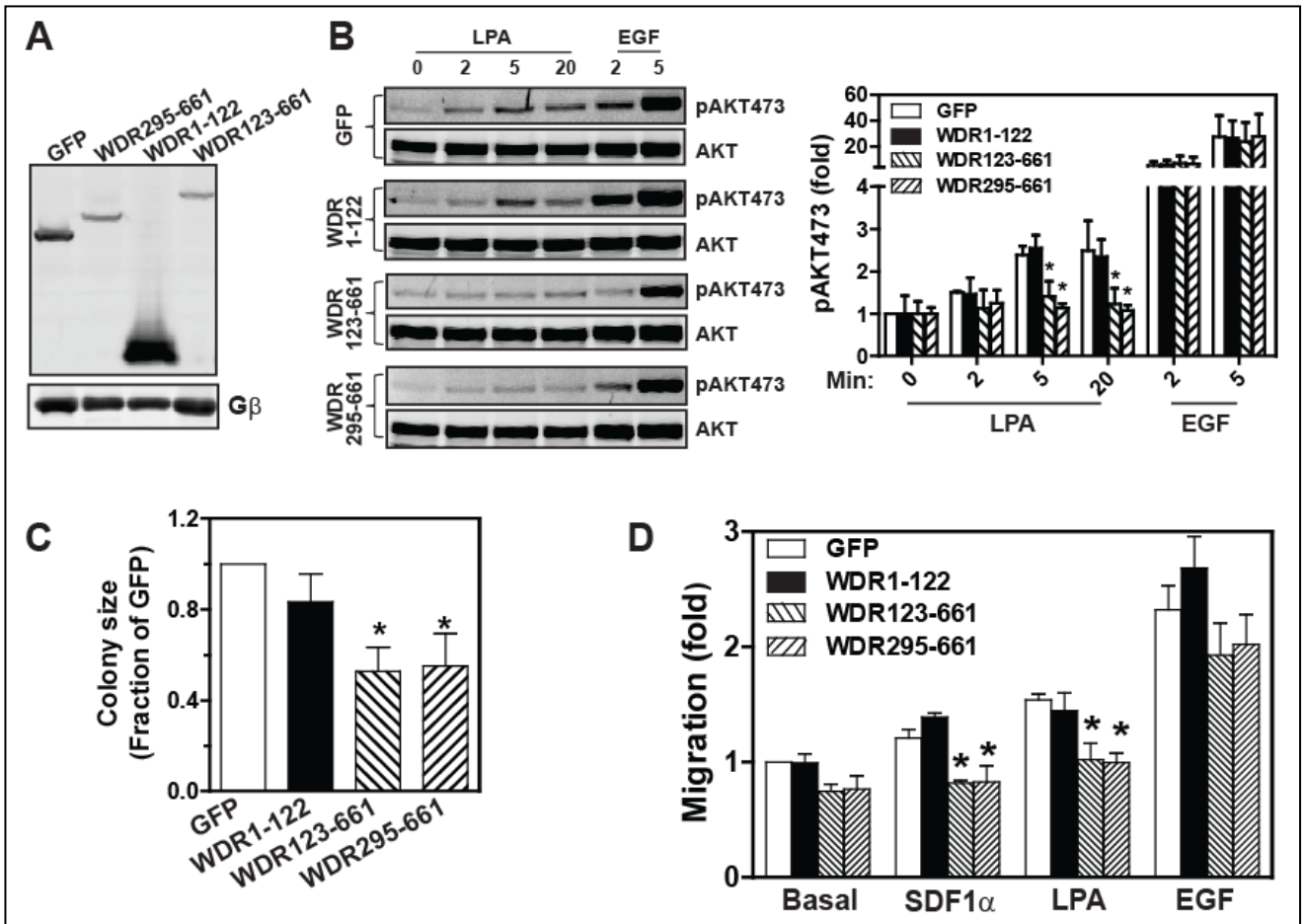

Figure 6. The effects of WDR26 mutants on AKT activation, tumor cell growth, and migration. A, Western blot analysis shows expression of GFP and WDR26 mutants in MDA-MB231 cells induced by a three-day treatment with doxycycline. B, LPA- and EGF-stimulated AKT phosphorylation in MDA-MB231 cells expressing the indicated constructs (n=4). C-D, effects of overexpressing WDR26 mutants on MDA-MB231 cell growth in Matrigel (C) and migration (D). * $p<0.05$ *versus* GFP (n=3-4).

4. **Impact:** our results thus far identify a novel mechanism regulating GPCR-dependent activation of the PI3K/AKT signaling axis in breast tumor cells, and pinpoint WDR26 as a potential therapeutic target for breast cancer.
5. **Change or problems:** nothing to report.
6. **Products:** nothing to report.
7. **Participants & other collaborating organizations:**
   Yuanchao Ye, PhD, a postdoctoral fellow, has been working on this project for the last 12 months. She has performed the work described here.
8. **Special Reporting Requirements: n/a**
9. **Appendices: none**